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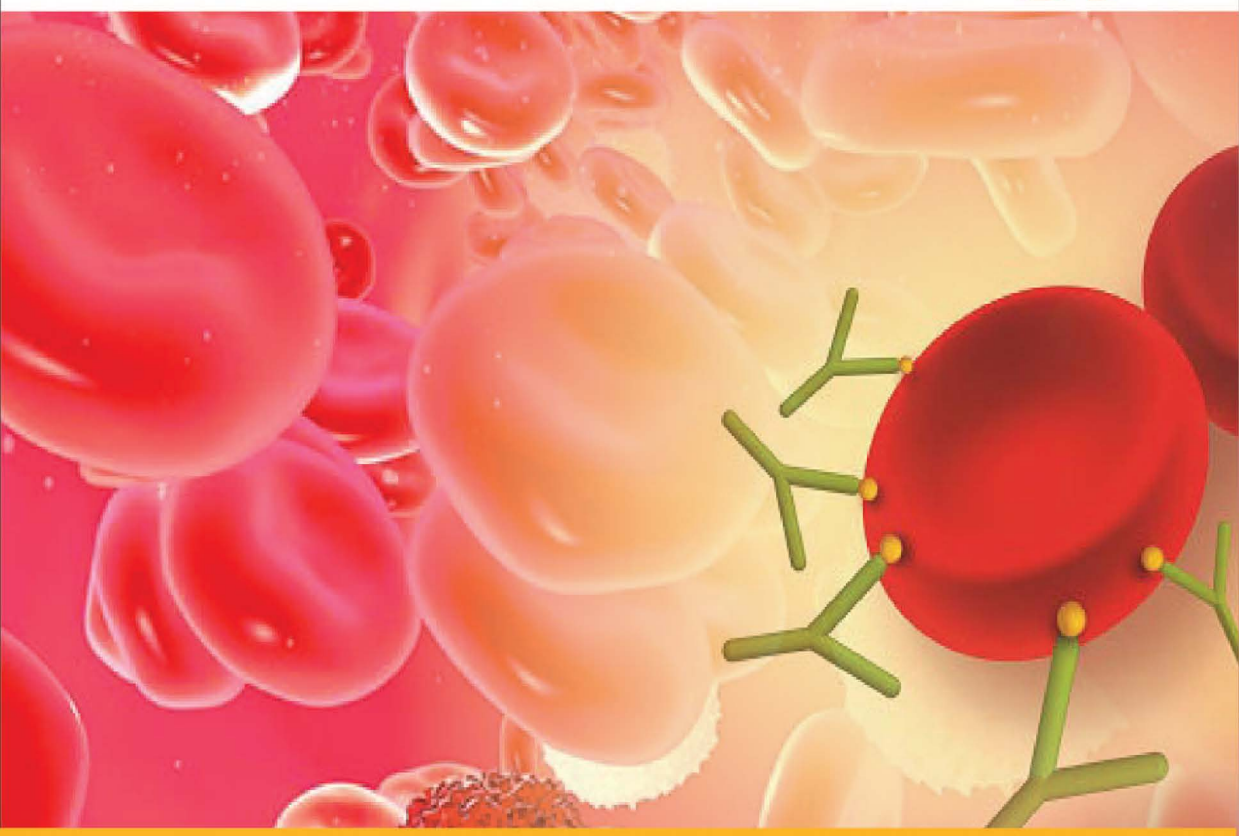
ICMR-NIIH Practical Guide to Laboratory Immunohematology



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Section 4

Transfusion Transmitted Disorders

Aruna Shankarkumar
Bhavik Dalal

4.1. Hepatitis C Virus Viral Load
4.2. Hepatitis C Virus Genotyping
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4.8. HBeAg ELISA

The most common transfusion-associated viral infections are human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2), hepatitis B virus (HBV), hepatitis C virus (HCV). Various methods with different sensitivity and specificity are commercially available as screening and confirmatory tests, not only for diagnosis but also to monitor disease progression and response to therapy.

The need for quantification of viral nucleic acids often referred to as 'viral load testing' in biological fluids have shown increasing demand over the past several years. It is a good surrogate marker for the progression of the disease. Assays based on the detection of viral nucleic acids have proved more effective than other conventional techniques. Methods for detection include quantitative real-time polymerase chain reaction (RT-PCR), branched DNA (bDNA) technology, nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA) and the SHARP Signal System. Understanding the natural history and pathogenesis of virus infections such as HIV, HBV and HCV have been greatly enhanced by determination of viral load *(Berger et al. 2001, Sacks et al. 2004*). Quantification of viral load can be used to assess disease progression, efficacy of therapy and also the emergence of drug-resistant viruses (*Preiser 2000*).

4.1 Hepatitis C Virus Viral Load

SAMPLE COLLECTION

- Blood should be collected in ethylenediaminetetraacetic acid (EDTA) or plain vacutainers
- This may be stored at 2–25°C up to maximum 6 hours
- Separate serum or plasma from whole blood into a sterile polypropylene tube.

PRINCIPLE

There are three important steps:

1. Specimen preparation to extract HCV RNA
2. Reverse transcription of the target RNA to generate complementary DNA (cDNA)
3. Polymerase chain reaction amplification of target cDNA using complementary primers specific to HCV and simultaneous detection of HCV target amplicon using oligonucleotide probes which are labeled with dual fluorescent dyes. The master mix contains primer pairs and probes specific for both HCV RNA and HCV standard RNA allowing independent detection of HCV amplicon and quantitation standard amplicon. The quantitation standard of HCV is a noninfectious armored RNA construct that contains the HCV sequences with similar primer binding sites as

the HCV RNA target and a unique probe binding region that enables HCV quantitation standard amplicon to be distinguished from HCV target amplicon. The HCV quantitation standard is integrated into each individual control and specimen at a known copy number throughout the RT-PCR steps together with the HCV target. The calculation of the HCV RNA titer in the test specimen is done by comparing the HCV signal to the HCV quantitation standard signal for each specimen and control. The HCV quantitation standard compensates for effects of inhibition to allow the accurate quantitation of HCV RNA in each specimen.

Note:

- Allow reagents to reach ambient temperature before proceeding
- Preheat heating block(s) to a temperature of 70°C (± 2°C) and water bath to a temperature of 50°C (± 2°C) before starting the purification reactions
- If using a frozen serum or plasma specimens, place the specimens at RT until completely thawed and vortex for 5–10 seconds before use.

4.2 Hepatitis C Virus Genotyping

The most commonly used method exploits the TaqMan assay principle. The forward and reverse primers hybridize to a specific sequence during PCR. A TaqMan probe, which is present in the same reaction mixture consisting of an oligonucleotide labeled with a 5'-reporter dye and a downstream 3'-quencher dye hybridizes to a target sequence within the PCR product. The Taq polymerase which possesses 5'–3' exonuclease activity cleaves the probe. The reporter and quencher dyes are separated upon cleavage resulting in an increase in fluorescence. The increase in fluorescence is directly proportional to the target amplicon.

SPECIMEN STORAGE

- After centrifugation, storage of plasma or serum can be done for up to 6 hours at 2–8°C
- Freezing aliquots at –20°C to –80°C storage conditions is recommended for long-term stability
- Frozen plasma or serum samples should not be thawed more than once. Doing so will lead to denaturation and precipitation of proteins causing reduced viral titers and subsequently reduced yields of the isolated viral RNA.

PRINCIPLE

Hepatitis C virus does not exist in the form of a "homogeneous" species. Due to elevated error rates in RNA replication, heterogeneous genomes or

"quasispecies" arising from mutations are discovered within the same host. Many significant biological characteristics of viruses are attributable to the nature of these quasispecies, including failure of vaccination, constant infection and antiviral resistance. It has also been discovered that the magnitude of diversity is associated with the progression to liver disease.

The capacity of the virus to persist in the host is the most striking characteristic of the HCV virus. The underlying mechanism of viral persistence is not clear. It is unable to incorporate itself into the host genome because of the unavailability of a DNA intermediate in viral life cycle. Instead, longevity tends to arise from the capacity of the virus to mutate quickly under immune challenge resulting in immunologically different species. Any of these can become the predominant type and the coexistence of various quasispecies enables HCV to bypass the host immune response.

Most mutations occur in a short, highly variable region in the E2/NS1 of the viral genome. This area constitutes only 8% of the genome, but is accountable for roughly half of the nucleotide modifications in the complete envelope region. Nucleotide replacement rates in the hypervariable region are noted to increase during acute infection when serum HCV RNA concentrations are declining, potentially owing to host immune response.

Viruses have been conventionally categorized according to antigenic features, but with latest technological advances in molecular biology, determination of genome through genomic variation analysis is now feasible. The sequence of the 5' NC region is comparatively conserved among the various HCV genotypes and is most frequently chosen for diagnosis. On the other hand, the sequences of NS3, NS5, and core regions are more variable and are therefore often used to define and distinguish HCV genotypes. There are nine significant HCV types identified as 1 through 9. Some of these kinds are further broken down into subtypes.

Around 170 million individuals globally are projected to be affected by chronic HCV disease and about 20–30% of these instances will ultimately advance to liver cirrhosis and further into developing hepatocellular carcinoma. Many studies have shown that interferon and ribavirin combinations are more efficient in treating HCV infection than interferon-only monotherapy. Therefore, it is essential to determine the HCV genotype before treatment because it has consequences for treatment, governance and therapeutic reaction. In addition, HCV genotype assays may be particularly helpful in understanding the local and global evolution since HCV's epidemiology is quickly evolving.

The HCV genotyping assay is designed for laboratory scale or high throughput transcript analysis by real-time PCR fluorescence. The genotyping kit identifies and differentiates the most common HCV genotypes, i.e. 1, 2, 3 and 4.

4.3 Anti-HBc ELISA

The hepatitis B core antigen (HBc) enzyme-linked immunosorbent assay (ELISA) is based on the principle of competitive ELISA. Anti-HBc IgG and IgM antibodies if present in the sample compete with a virus-specific monoclonal IgG, labeled with horseradish peroxidase (HRP) for a fixed amount of recombinant HBcAg purified and coated on the microplate. The competitive assay is carried out in one reaction.

The concentration of the bound enzyme on the solid phase becomes inversely proportional to the amount of anti-HBc in the sample and its activity is detected by adding the chromogen/substrate solution in the second incubation. The concentration of antibodies to HBcAg in the sample is determined by means of a cutoff value that allows for the semiquantitative detection of anti-HBc.

4.4 Human Immunodeficiency Virus Viral Load

SAMPLE COLLECTION, PROCESSING AND STORAGE

- Peripheral blood is collected in K_2EDTA tubes by standard venipuncture using preferably a large gauge needle
- Avoid using heparinized blood as it inhibits the PCR reaction
- Cap and swirl the tubes for uniform mixing
- When using K_2EDTA, it is possible to collect whole blood in tubes with or without a separator of gel. In the case of a nongel separator tube, the K_2EDTA blood samples are centrifuged for 20 minutes at 1,000–1,500 g to separate plasma from cells
- If blood samples are frozen or stored for a longer period of time, the sensitivity of the assay may be reduced
- Separated plasma can be transferred in situ to the laboratory in a gel separator tube
- Plasma must be stored at –20°C as it is stable at 2–8°C for only five days and longer if frozen at or below –20°C or –70°C
- Do not store plasma samples in the freezer compartment of a refrigerator as the temperature in this type of freezer is cycled several times a day, causing nucleic acid targets to degrade.

VIRAL RNA EXTRACTION

Most commercially accessible devices for the extraction of nucleic acid use silica-membrane technology and offer an easy way to remove viral RNA and DNA from serum or plasma specimens concurrently.

Amplification: Real-Time PCR

The viral load assay kit is used to detect the genome of HIV genotypes (A, B, C, D, AE, F, G, AA-GH) and quantify HIV-1 genome in plasma, using RT-PCR. The viral load is measured using four quantification standards provided in the kit. Both the clinical picture and laboratory markers are used to monitor HIV infection.

Human plasma is used for quantitation of HIV (A, B, C, D, AE, F, G, AA-GH subtypes). The set includes the reagents needed to perform RT-PCR for HIV quantitation. The PCR amplifies only a specific region of viral genome (i.e. gag). In addition, the kit contains a second amplification system to identify possible PCR inhibition by using an internal control (IC) without affecting the analytical sensitivity of the assay. Other external quantitation standards calibrated with World Health Organization (WHO) control are also used for quantification of HIV RNA.

4.5 Hepatitis B Virus DNA Quantitation

Hepatitis B virus (HBV) is a partially double stranded DNA virus that belongs to the family Hepadnaviridae. Approximately 350 million people are chronically infected with HBV worldwide, placing them at high risk for developing cirrhosis, end-stage liver disease, and hepatocellular carcinoma. Detection and quantification of circulating HBV in the blood plays a vital role in monitoring and diagnosing HBV infection as well as assessing response to antiviral therapy. Clinically, HBV DNA titers vary greatly with levels as high as 10^{10} copies/mL during acute HBV infection to very low levels in hepatitis B e-antigen-negative chronic carriers, in patients undergoing antiviral therapy and in those with occult HBV infection. The most commonly used method for HBV DNA quantification is based on real-time PCR technology which offers the great sensitivity and broad linear dynamic range.

4.6 Anti-HBs Quantitative ELISA

The microplate wells are coated with hepatitis B surface antigen (HBsAg) that specifically captures anti-HBs during the first incubation step. After a thorough washing step, captured antibodies are detected by HBsAg labeled with an enzyme that binds the available binding sites of these antibodies. The enzyme bound to the antibodies generates an optical signal in the presence of chromogen/substrate. The signal produced is proportional to the amount of Anti-HBs in the sample and can be estimated using a photometric microplate

reader. The quantitation of antibodies may be done by means of a calibration curve.

4.7 Hepatitis B Surface Antigen ELISA

The HBsAg ELISA is generally based on the principle of sandwich ELISA. The microplate for the quantitative detection is precoated with anti-hepatitis B surface antigen (anti-HBs). The HBsAg in the serum binds to anti-HBs, followed by a second antibody-conjugate forming a sandwich. After washing, conjugate bound to the secondary antibody reacts with substrate solution producing a colored product which can be read using a photometric microplate reader. The intensity of the color is directly related to the quantity of HBsAg present in the serum of the patient.

4.8 HBeAg ELISA

The hepatitis B e-antigen (HBeAg) assay is generally based on the principle of sandwich ELISA. The solid phase is coated with a specific monoclonal anti-HBe that captures the HBe antigen if present in the sample. Subsequently, a second monoclonal antibody labeled with the conjugate binds the captured antigen. In the third step, the enzyme bound to the monoclonal antibody interacts with the chromogen/substrate generating a colored product that can be detected using a spectrophotometer. The intensity of the color is proportional to the concentration of the antigen in the sample.

ICMR-NIIH Practical Guide to Laboratory Immunohematology

Salient Features

- A comprehensive practical laboratory guide for both medical and paramedical practitioners.
- Comprehensive coverage of laboratory techniques of a wide range of immunohematological disorders.
- Genetic diagnosis of monogenic and multigenic disorders with special emphasis on carrier diagnosis and antenatal diagnosis.
- Step-by-step procedures and readily reproducible techniques.
- Diagnostic algorithms for complex disorders with multiple etiologies like primary immunodeficiencies, hemoglobinopathies, and disorders of hemostasis.
- Simplified in-house techniques, quality control exercises, and notes on troubleshooting.
- Classical and unusual case illustrations.

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